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VACCINES AND ANTIVIRAL AGENTS

Novel Insect-Specific Eilat Virus-Based Chimeric Vaccine Candidates Provide Durable, Mono- and Multivalent, Single-Dose Protection against Lethal Alphavirus Challenge

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ABSTRACT Most alphaviruses are mosquito borne and exhibit a broad host range, infecting many different vertebrates, including birds, rodents, equids, humans, and nonhuman primates. Recently, a host-restricted, mosquito-borne alphavirus, Eilat virus (EILV), was described with an inability to infect vertebrate cells based on defective attachment and/or entry, as well as a lack of genomic RNA replication. We investigated the utilization of EILV recombinant technology as a vaccine platform against eastern (EEEV) and Venezuelan equine encephalitis viruses (VEEV), two important pathogens of humans and domesticated animals. EILV chimeras containing structural proteins of EEEV or VEEV were engineered and successfully rescued in *Aedes albopictus* cells. Cryo-electron microscopy reconstructions at 8 and 11 Å of EILV/VEEV and EILV/EEEV, respectively, showed virion and glycoprotein spike structures similar to those of VEEV-TC83 and other alphaviruses. The chimeras were unable to replicate in vertebrate cell lines or in brains of newborn mice when injected intracranially. Histopathologic examinations of the brain tissues showed no evidence of pathological lesions and were indistinguishable from those of mock-infected animals. A single-dose immunization of either monovalent or multivalent EILV chimera(s) generated neutralizing antibody responses and protected animals against lethal challenge 70 days later. Lastly, a single dose of monovalent EILV chimeras generated protective responses as early as day 1 postvaccination and partial or complete protection by day 6. These data demonstrate the safety, immunogenicity, and efficacy of novel insect-specific EILV-based chimeras as potential EEEV and VEEV vaccines.

IMPORTANCE Mostly in the last decade, insect-specific viruses have been discovered in several arbovirus families. However, most of these viruses are not well studied and largely have been ignored. We explored the use of the mosquito-specific alphavirus EILV as an alphavirus vaccine platform in well-established disease models for eastern (EEE) and Venezuelan equine encephalitis (VEE). EILV-based chimeras replicated to high titers in a mosquito cell line yet retained their host range restriction in vertebrates both *in vitro* and *in vivo*. In addition, the chimeras generated immune responses that were higher than those of other human and/or equine vaccines. These findings indicate the feasibility of producing a safe, efficacious, mono- or multivalent vaccine against the encephalitic alphaviruses VEEV and EEEV. Lastly, these data demonstrate how host-restricted, insect-specific viruses can be engineered to develop

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vaccines against related pathogenic arboviruses that cause severe disease in humans and domesticated animals.

KEYWORDS alphavirus, emerging infectious diseases, vaccines, Eilat virus

The genus *Alphavirus* in the family *Togaviridae* is composed of small, spherical, enveloped viruses with genomes consisting of a single-stranded, positive-sense RNA 11 to 12 kb in length. Alphaviruses comprise 31 recognized species classified into 11 complexes based on antigenic and/or genetic similarities (1–3). The two aquatic alphavirus complexes are not known to utilize arthropods in their transmission cycles, whereas all of the remaining complexes (Barmah Forest; Ndumu; Middelburg; Semliki Forest; Venezuelan [VEE], eastern [EEE], and western equine encephalitis [WEE]; Trocara; and Eilat) consist of arboviruses that almost exclusively utilize mosquitoes as vectors (1–3). Mosquito-borne alphaviruses infect diverse vertebrate hosts, including equids, birds, amphibians, reptiles, rodents, pigs, humans, and nonhuman primates (1).

The ability to infect both mosquitoes and vertebrates enables the maintenance of alphaviruses in natural endemic transmission cycles that occasionally spill over into the human population and cause disease. Infections with Old World alphaviruses, such as chikungunya, o'nyong-nyong, Sindbis, and Ross River, are rarely fatal, but disease is characterized by rash and debilitating arthralgia that can persist for months or years. In contrast, New World alphaviruses, such as eastern (EEEV), western (WEEV), and Venezuelan equine encephalitis virus (VEEV), can cause life-threatening encephalitis with case fatality rates of 40 to 70% for EEEV and $<$1 to 5% for WEEV and VEEV infections, and many survivors suffer debilitating, permanent neurologic sequelae (1). In addition, all three viruses can also cause fatal disease in equids, with case fatality rates ranging from 20 to 90% (1).

Due to the potential for periodic outbreaks and fatal disease, encephalitic alphaviruses are important human and domesticated animal pathogens. In an effort to combat both EEEV and VEEV infections, live-attenuated VEEV-IAB TC-83 and formalin-inactivated vaccines were prepared from wild-type EEEV (PE6) and TC-83 (C84) and developed under Investigational New Drug (IND) status (4–8). However, these vaccines are only available for limited use in laboratory and military personnel as well as in licensed forms for domesticated animals. In addition, these vaccines suffer from high rates of reactogenicity (TC-83), risk of residual live virus in the vaccine lot, and poor immunogenicity (C84 and PE-6) (9, 10). Currently, there are no licensed antiviral treatments or vaccines to prevent or treat EEEV or VEEV infection, and the U.S. population remains vulnerable to natural disease outbreaks.

Recently, a host-restricted alphavirus, Eilat virus (EILV), was described that is serologically distinct but phylogenetically groups within the mosquito-borne clade as a sister to the WEE complex (3). However, in contrast to all other mosquito-borne alphaviruses, EILV is unable to infect and replicate in vertebrate cells but can readily replicate to high titers ($>10^8$ PFU/ml) in mosquito cells (3). Investigation into the nature of EILV vertebrate host range restriction demonstrated that blocks at both genomic RNA replication and attachment and/or entry levels involve at least two genes (11). The successful rescue in mosquito cells of EILV chimeras containing Sindbis virus (SINV) structural genes, which were unable to replicate in vertebrate cells, raised an intriguing possibility to utilize EILV chimeras as a possible vaccine platform for pathogenic alphaviruses (11). Following the first use of EILV as a vaccine for chikungunya fever, we investigated the utilization of EILV-based vaccines against EEEV and VEEV in a lethal murine challenge model (12).

RESULTS

Generation, *in vitro* characterization, and cryo-EM of EILV chimeras. EILV chimeras were engineered to express the structural polyprotein open reading frame (ORF) of EEEV-FL93 (EILV/EEEV) and VEEV-IAB TC-83 (EILV/VEEV) (Fig. 1). In addition, reporter constructs expressing either enhanced red fluorescent protein (EILV/EEEV eRFP) or

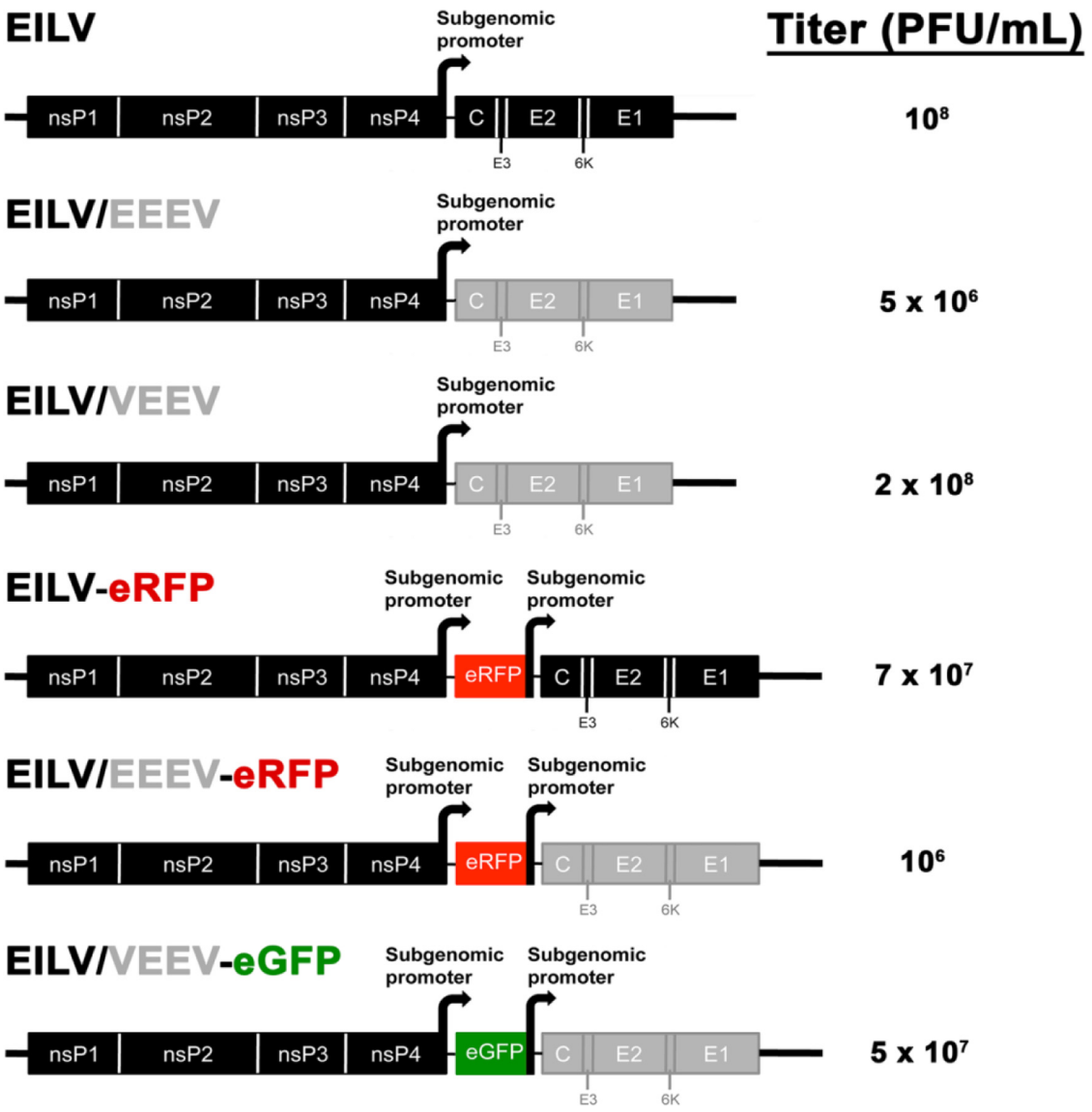

FIG 1 Schematic diagrams of EILV chimeras. EILV chimeras were rescued in C7/10 cells, and 48 h postelectroporation (hpe) supernatants were harvested and titrated on C7/10 cells.

enhanced green fluorescent protein (EILV/VEEV eGFP) under the control of a second subgenomic promoter were engineered (Fig. 1). All chimeras were readily rescued in C7/10 cells within 48 h postelectroporation (hpe), with titers ranging from 10^6 to 10^8 PFU/ml, with EILV/EEEV chimeras yielding lower titers (Fig. 1).

To determine whether virion and glycoprotein spike structures of EILV chimeras were similar to those of other alphaviruses, cryo-electron microscopic (cryo-EM) imaging and reconstruction was performed. Similar to those of other alphaviruses, both EILV/EEEV and EILV/VEEV chimeric virions were spherical and ~70 nm in diameter (Fig. 2A and C). EILV/EEEV and EILV/VEEV cryo-EM reconstruction at 11- and 8-Å-resolution, respectively, showed that the surface spike structures were very similar to those published for VEEV and other alphaviruses (Fig. 2B and D) (13, 14). Surprisingly, long-term storage of chimeras did not change particle morphology, as aliquots of EILV chimeras were stored at 4°C for 1 to 2 years and did not show any changes in particle appearance (Fig. 3).

Host range restriction of EILV chimeras *in vitro* and *in vivo*. To ensure that the EILV chimeras retained the host-restricted phenotype in vertebrate cells, EILV/EEEV and

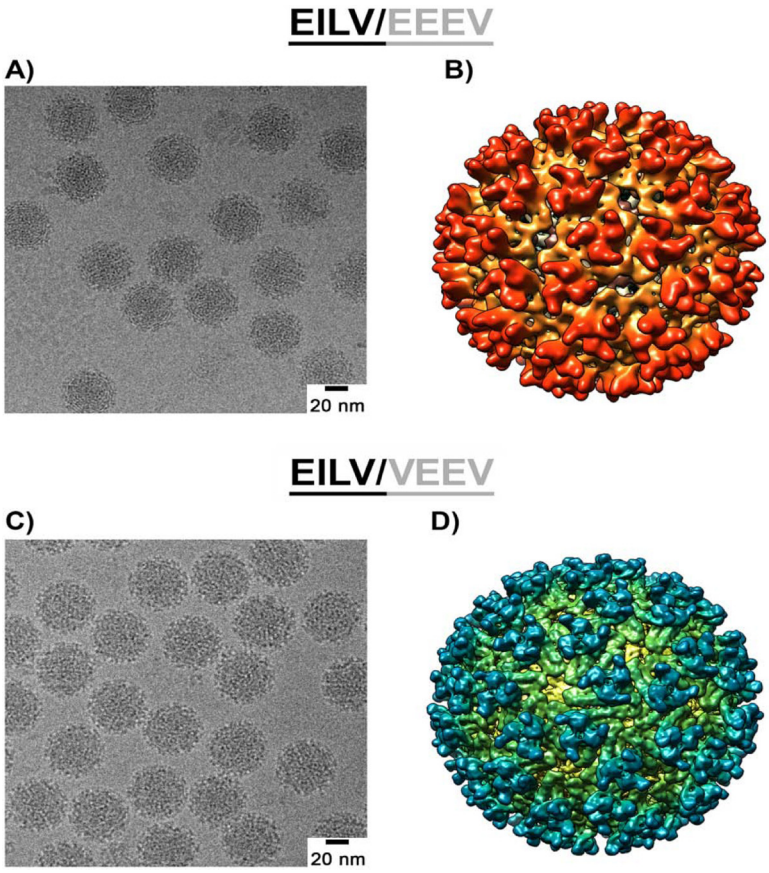

FIG 2 Virion morphology of EILV chimeras determined by cryo-electron (cryo-EM) microscopy. Cryo-EM micrographs of EILV/EEEV (A) and EILV/VEEV (B). An 11-Å and 8-Å cryo-EM single-particle reconstruction of EILV/EEEV (B) and EILV/VEEV (D).

EILV/VEEV, as well as reporter constructs expressing EILV/EEEV eRFP and EILV/VEEV eGFP, were *in vitro* transcribed, and capped RNA was electroporated into Vero, HEK-293, and BHK-21 vertebrate cell lines (Fig. 4). Neither infectious virus nor reporter expression could be detected up to 4 days postelectroporation (Fig. 4). In contrast, infectious virus and reporter expression could be readily detected within 24 hpe or hours postinfection (hpi) in mosquito cells (Fig. 4).

The host range restriction of EILV chimeras next was investigated *in vivo* in CD-1 mice. Seven-day-old animals were injected intracranially with 10^8 PFU of EILV/EEEV or EILV/VEEV. VEEV-IAB TC-83 vaccine at 10^4 PFU/animal and C7/10 supernatants were utilized as positive and negative controls, respectively (Fig. 5A). Animals were serially

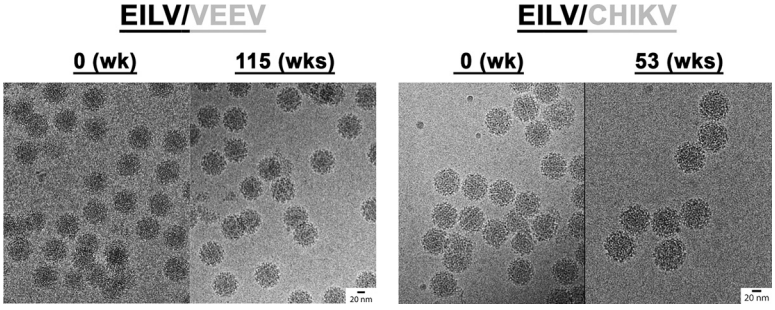

FIG 3 (A) Micrographs of EILV/VEEV were taken after 0 weeks (left) and 115 weeks (right) of storage at 4°C. (B) Micrographs of EILV/CHIKV were collected after 0 weeks (left) and 53 weeks (right) of storage at 4°C.

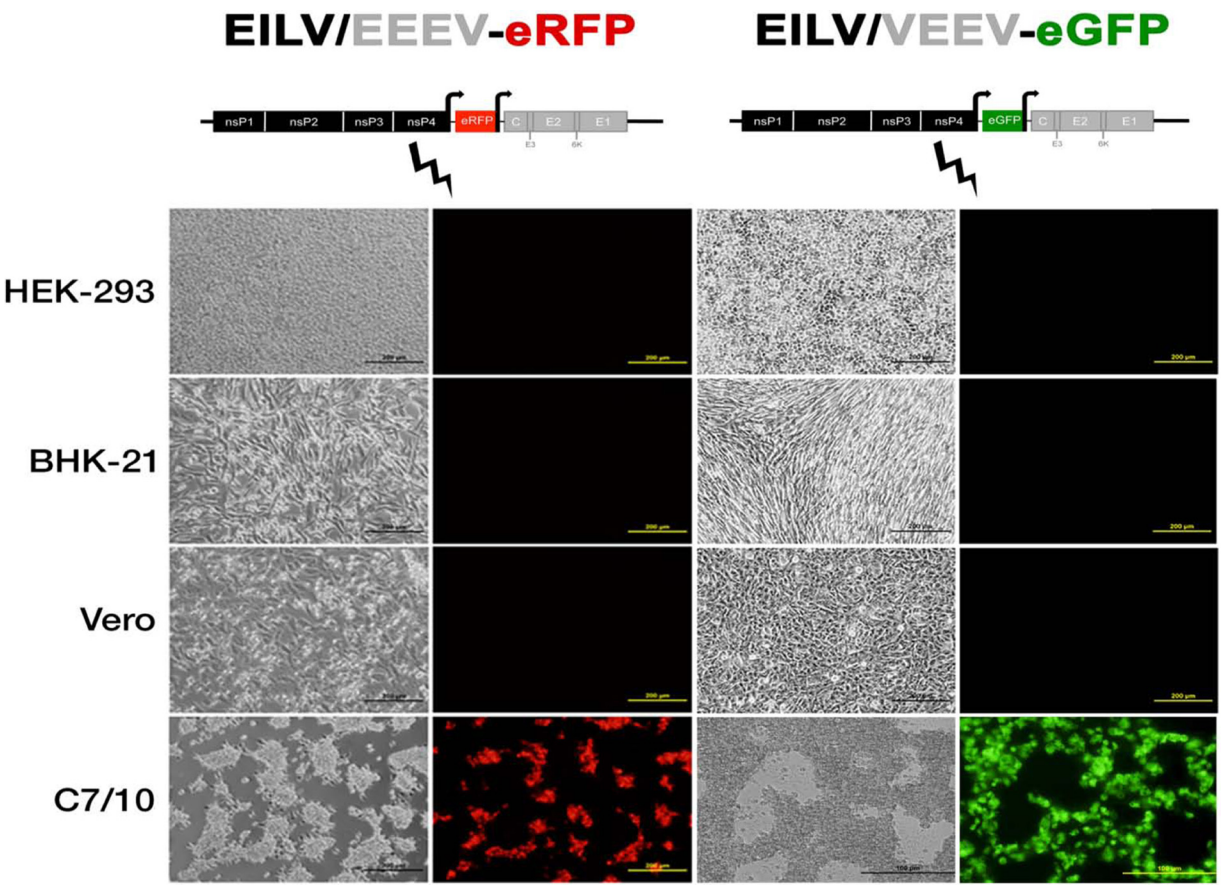

FIG 4 Electroporation of vertebrate and mosquito cell lines with EILV reporter chimeras. RNA was transcribed *in vitro*, each cell line was electroporated with ~10 μg of RNA, and phase-contrast (left) and fluorescence (right) micrographs were taken at 1 and 4 days postelectroporation (dpe).

sacrificed to determine virus replication and potential neurovirulence of the EILV chimeras (Fig. 5B and C). The highest titers of both EILV chimeras were measured at day 0 postinoculation and declined precipitously to below the limit of detection by days 3 and 7 post-intracranial injection (Fig. 5C). All animals injected with EILV chimeras survived until the end of the study (Fig. 5B). In addition, no brain lesions were detected at any time postinfection, and the brain sections of injected animals were indistinguishable from those of the C7/10 supernatant control animals (Fig. 6). In contrast, VEEV-IAB TC-83 was able to replicate to high titers ($>10^8$ PFU/g) by day 3 postinjection, and all animals met the euthanasia criteria by day 13 postinoculation (Fig. 5B and C). The brain tissues displayed extensive necrotic and inflammatory lesions in all animals (Fig. 6).

Assessment of monovalent EILV/EEEV and EILV/VEEV vaccine vector efficacy in lethal alphavirus challenge. The efficacy of both EILV/EEEV and EILV/VEEV vaccine candidates was assessed in a lethal murine challenge model. Cohorts of 5 outbred CD-1 mice were mock vaccinated or vaccinated with EILV/EEEV at 10^8 PFU/mouse via the subcutaneous (s.c.) route (Fig. 7A). A commercially available, formalin-inactivated EEEV equine vaccine at one-tenth the recommended dose for equids was utilized as a positive control (Fig. 7A). Neutralizing antibody responses were measured via 80% plaque reduction neutralization tests ($PRNT_{80}$) at 6, 28, and 65 days postvaccination. Neutralizing antibodies could be detected by 6 days postvaccination with EILV/EEEV, with $PRNT_{80}$ titers of 1:20 and 1:40 detected in 60% of the mice (Fig. 7B). The seroconversion rates at days 28 and 65 were 100%, with $PRNT_{80}$ titers ranging from 1:80 to 1:640 (Fig. 7B). In contrast, neutralizing antibodies could not be detected in mice

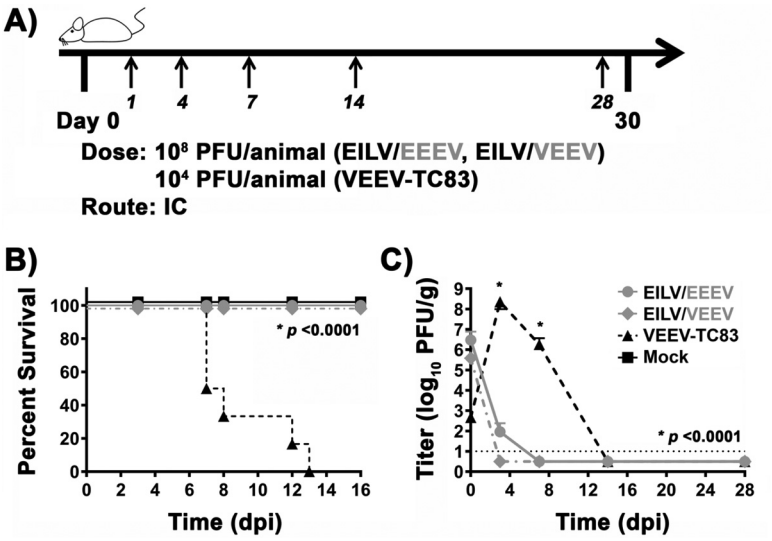

FIG 5 Neurovirulence of EILV chimeras in newborn mice. (A) Outline of the experimental design. (B and C) Percent survival (B) and virus replication (C) following intracranial inoculation of EILV chimeras, VEEV-TC83, and sucrose-purified C7/10 cell supernatants. Average virus titers are shown with each data point representing brain homogenate from 3 animals ± standard deviations (SD). *, $P < 0.0001$.

vaccinated with the commercial horse vaccine at day 6 (Fig. 7B). The seroconversion rates were 100% and 80% at 28 and 65 days postvaccination, respectively, and the average $PRNT_{80}$ titers were 2- to 3-fold lower than those following EILV/EEEV vaccination at both time points, with titers ranging from 1:20 to 1:160 (Fig. 7B). Animals were challenged 70 days postvaccination with lethal EEEV-FL93 at 10^5 PFU/animal (100% lethal dose [LD_{100}]) via the intraperitoneal (i.p.) route. Little or no weight loss was observed in mice that received either vaccine, and all survived (Fig. 7C and D). In contrast, all mock-vaccinated animals lost weight postchallenge, displayed signs of severe illness (loss of coordination, hind limb paralysis, and seizures), and met euthanasia criteria by day 5 postchallenge (Fig. 7C and D).

The efficacy of EILV/VEEV next was assessed in a lethal murine challenge model. Cohorts of 5 CD-1 mice were mock vaccinated or vaccinated with EILV/VEEV at 10^8 PFU/mouse via the s.c. route (Fig. 8A). VEEV-TC83 and a commercial, formalin-inactivated equine VEEV vaccine at one-tenth the recommended dose for equids were utilized as positive controls. Neutralizing antibody could be detected at 6, 28, and 65 days postvaccination in all mice vaccinated with EILV/VEEV, with $PRNT_{80}$ titers ranging from 1:80 to 1:1,280 (Fig. 8B). Animals vaccinated with VEEV-TC83 displayed a similar magnitude of neutralizing antibody response at 6 days postvaccination and a seroconversion rate of 60% (Fig. 8B). At 28 and 65 days postvaccination the TC-83 seroconversion rates were 80% and 60%, respectively. In addition, the average $PRNT_{80}$ titers were 2- to 8-fold lower than those following EILV/VEEV vaccination, with titers ranging from 1:20 to 1:640 (Fig. 8B). No neutralizing titers were observed in mice vaccinated with the commercial equine vaccine, which is typically administered as a two-dose regimen (Fig. 8B). At day 70, mice were challenged with VEEV-3908 at 10^3 PFU/mouse (LD_{100}) via the s.c. route. All animals vaccinated with EILV/VEEV or TC-83, regardless of seroconversion or magnitude of $PRNT_{80}$ titer, exhibited little or no weight loss and all mice survived (Fig. 8C and D). In contrast, all mock- and commercial vaccine-immunized mice displayed weight loss and clinical disease (loss of coordination, hind limb paralysis, and seizures) and met euthanasia criteria by day 9 postchallenge (Fig. 8C and D). These data demonstrated that monovalent EILV chimeras can protect mice against lethal VEEV and EEEV challenge following a single s.c. vaccination.

Efficacy of multivalent EILV/EEEV, EILV/VEEV, and EILV/CHIKV vaccine against lethal alphavirus challenge. To assess whether a single dose consisting of multiple

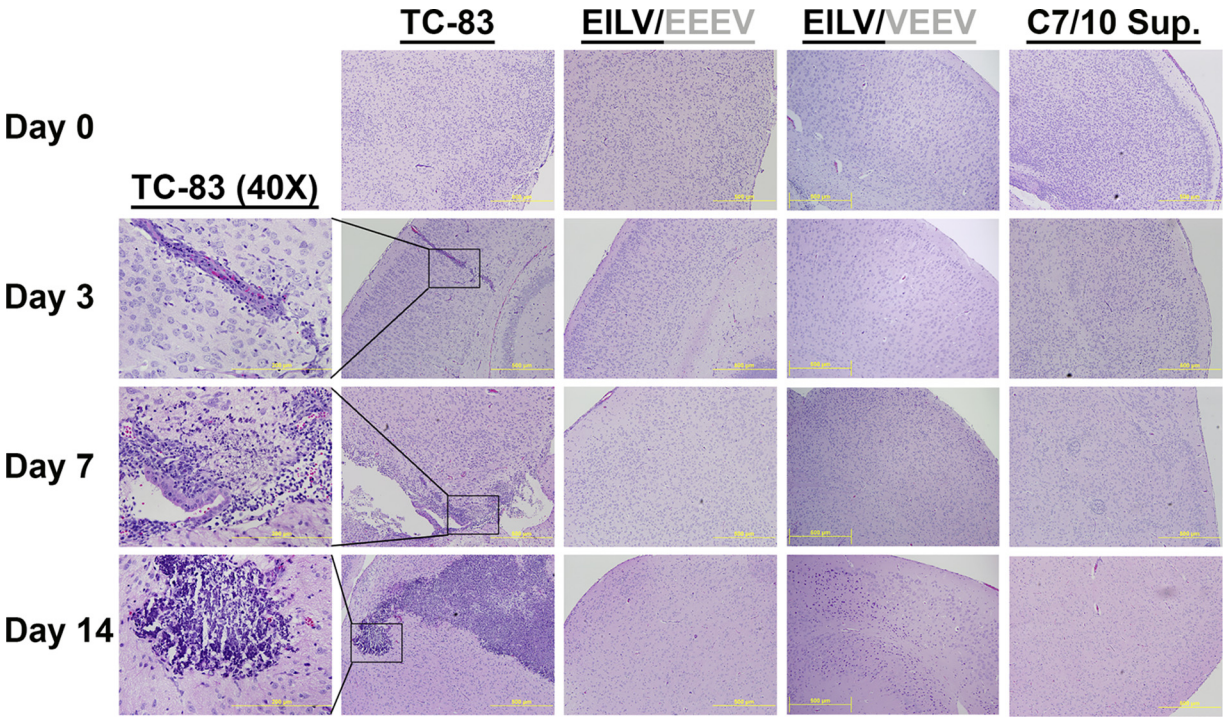

FIG 6 Histopathology of brain tissues. Micrographs of hematoxylin- and eosin-stained sections of brain tissues following intracranial inoculation of EILV chimeras, VEEV-TC83, and sucrose-purified C7/10 cell supernatants are shown. Three animals were sacrificed at the time points indicated, and tissues were stained to determine necrotic and inflammatory lesions. Necrotic and inflammatory lesions in VEEV-TC83 were magnified (40×) and are shown.

EILV chimeras could provide protection against lethal challenge with multiple alphaviruses, EILV/EEEV, EILV/VEEV, and the recently described EILV/CHIKV chimeras were blended into a trivalent formulation (12). Cohorts of 30 VEEV- or EEEV-infected CD-1 mice were either mock vaccinated or vaccinated s.c. with 10^8 PFU/each of monovalent or trivalent vaccine followed by lethal VEEV and EEEV challenge (Fig. 9A). Neutralizing antibody responses were measured via $PRNT_{80}$ assay at 28 days postvaccination against all three viruses. Animals receiving either the mono- or trivalent vaccine displayed similar anti-VEEV or -CHIKV neutralizing antibody response rates of >90%. The mean anti-VEEV $PRNT_{80}$ titer was 1:405 for both vaccine groups, with titers ranging from 1:40 to 1:640 and 1:20 to 1:640 for mono- and trivalent vaccines, respectively (Fig. 9B). The mean anti-CHIKV $PRNT_{80}$ titer was slightly lower for the trivalent vaccine group than that following monovalent vaccination at 1:80 versus 1:160, respectively (Fig. 9B). The $PRNT_{80}$ anti-CHIKV titers ranged from 1:80 to 1:320 and 1:40 to 1:160 for mono- and trivalent vaccines, respectively (Fig. 9B). In contrast, the neutralizing antibody response rate against EEEV in animals vaccinated with the trivalent vaccine was significantly lower than that in animals receiving monovalent vaccine at 50% versus 100%, respectively (Fig. 9B). Also, the average $PRNT_{80}$ titer of animals receiving trivalent vaccine was 8-fold lower than those receiving the monovalent vaccine, with titers for mono- and trivalent vaccines ranging from 1:40 to 1:640 and 1:20 to 1:80, respectively (Fig. 9B). Following lethal EEEV challenge, 70% of mock-vaccinated mice displayed clinical disease, lost weight, and met the euthanasia criteria by 7 days postchallenge. In contrast, only 20% of trivalent-vaccinated animals succumbed to infection (Fig. 9C). Similarly, following VEEV challenge, all mock-vaccinated mice met the euthanasia criteria by 6 days postchallenge, while 90% of trivalent-vaccinated animals survived (Fig. 9D). These data demonstrated that a single dose of blended trivalent EILV-VEEV, -EEEV, and -CHIKV vaccine can provide protection from lethal VEEV and EEEV challenge.

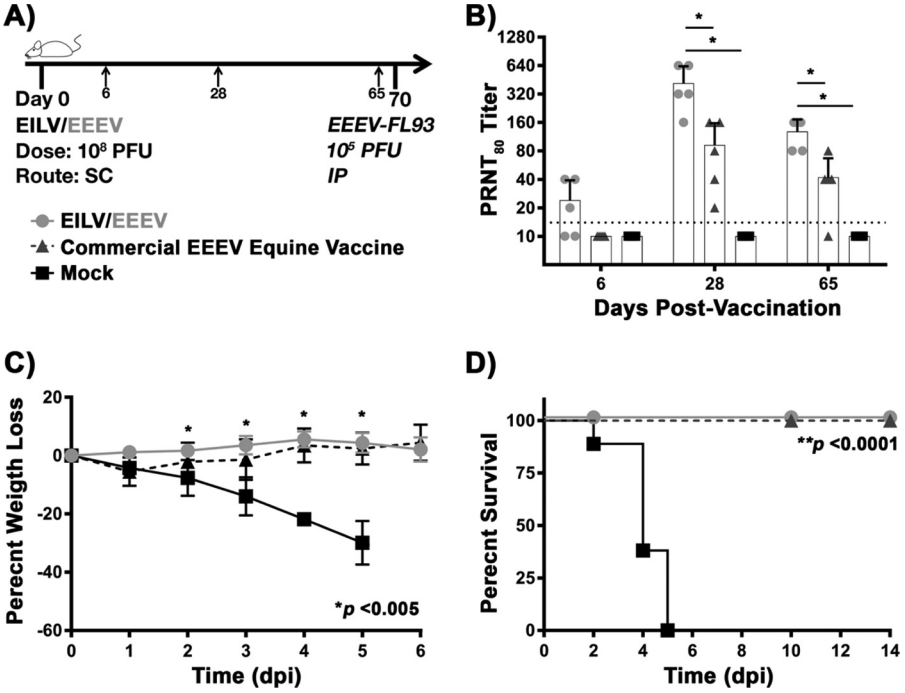

FIG 7 Immunogenicity and efficacy of monovalent EILV/EEEV chimera in CD-1 mice. (A) Outline of the experimental design. (B) Neutralizing antibody response measured by $PRNT_{80}$ titers following EILV/EEEV vaccination. Percent weight loss (C) and survival (D) following lethal EEEV-NA challenge via the subcutaneous route (SC). Average $PRNT_{80}$ titers and weight losses ± standard deviations (SD) (error bars) are shown. *, $P \leq 0.01$; **, $P < 0.0001$.

Investigating the kinetics of protective efficacy elicited by monovalent EILV/EEEV and EILV/VEEV vaccine. To determine the kinetics of the protective immune response elicited by EILV/EEEV and EILV/VEEV, cohorts of 40 or 50 CD-1 mice were vaccinated with either EILV/EEEV or EILV/VEEV at 10^8 PFU/ml, respectively, followed by lethal challenge at 1, 4, or 6 days postvaccination (Fig. 10A). Mock-vaccinated animals were used as negative controls. Animals vaccinated with EILV/EEEV were challenged with EEEV-FL93 at 10^5 PFU/animal via the i.p. route (Fig. 10A). Fifty percent of animals challenged at 6 days postvaccination displayed no clinical disease and survived, whereas 80% of animals challenged with lethal EEEV at 1 or 4 days postvaccination displayed clinical disease and weight loss and met the euthanasia criteria (Fig. 10B to D).

Similar to the EILV/EEEV study, cohorts of 50 CD-1 mice were vaccinated with EILV/VEEV at 10^8 PFU/ml, followed by lethal challenge at 1, 4, or 6 days postvaccination (Fig. 11A). VEEV-TC83- and mock-vaccinated animals were utilized as positive and negative controls, respectively (Fig. 11A). No animals vaccinated with either EILV/VEEV or VEEV-TC83 displayed clinical disease or weight loss, and all survived lethal VEEV challenge at 6 days postvaccination. In contrast, all mock-vaccinated animals displayed substantial disease (loss of coordination, hind limb paralysis, and seizures) and weight loss and met the euthanasia criteria by day 7 postchallenge (Fig. 11B). All EILV/VEEV-vaccinated and 80% of VEEV-TC83-vaccinated animals survived lethal VEEV challenge 4 days postvaccination (Fig. 11C). Surprisingly, 40% of the animals vaccinated with EILV/VEEV survived lethal VEEV challenge 1 day postvaccination (Fig. 11D). In contrast, all mock- and VEEV-TC83-vaccinated animals displayed clinical disease and weight loss and met the euthanasia criteria by day 7 or 10 postchallenge, respectively (Fig. 11D). These data demonstrated that a single monovalent dose of EILV chimeras can induce protective responses as early as 1 day postvaccination and can provide partial or complete protection by 6 days postvaccination.

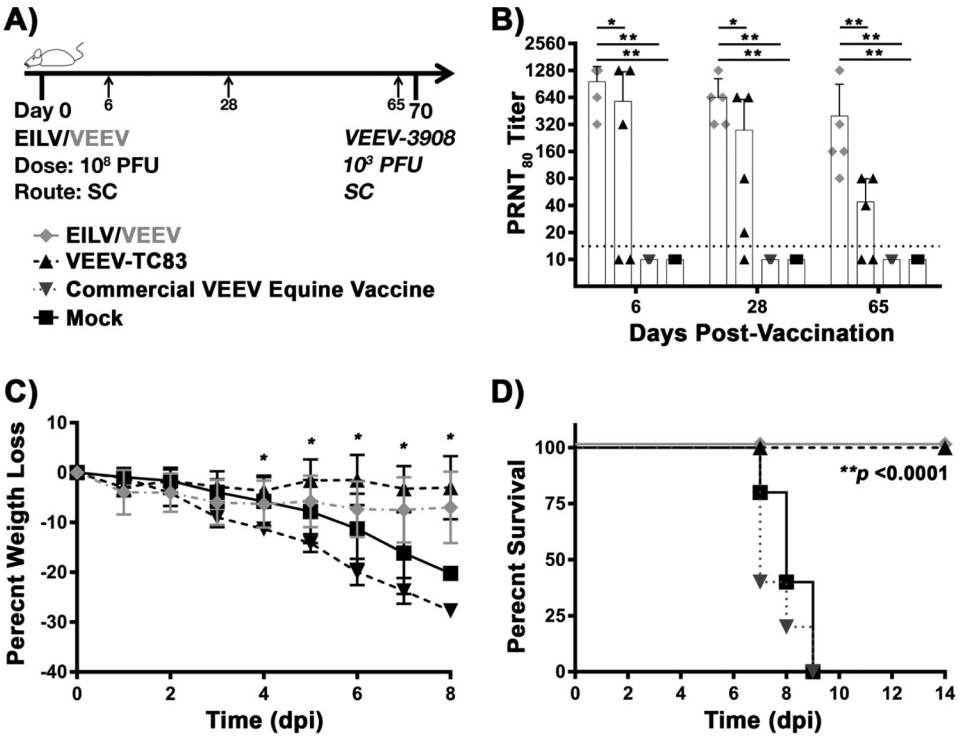

FIG 8 Immunogenicity and efficacy of monovalent EILV/VEEV chimera in CD-1 mice. (A) Outline of the experimental design. (B) Neutralizing antibody response measured by $PRNT_{80}$ titers following EILV/VEEV vaccination. (B and C) Percent weight loss (C) and survival (D) following lethal VEEV-IC challenge via the subcutaneous route (SC). Average $PRNT_{80}$ titers and weight losses ± SD (error bars) are shown. *, $P \leq 0.04$; **, $P < 0.0001$.

DISCUSSION

In order to propagate, viruses must enter susceptible cells to deliver their genome, hijack cellular machinery to facilitate their replication, and package new genomes to produce infectious progeny. Each of these steps constitutes a fundamental barrier that arboviruses must overcome in order to replicate in diverse arthropod and vertebrate hosts for transmission in nature; failure to replicate in either host would result in extinction. Arthropod- or insect-specific viruses are unable to overcome these critical barriers and therefore are unable to replicate in the vertebrate host. The recently characterized insect-specific EILV is vertebrate host restricted at attachment/entry as well as genomic RNA replication levels (11). In addition, the host range restriction determinants are multigenic, likely due to specialization for replication in insects that resulted in a loss of vertebrate replication competence or niche adaptation (11). The nature of the EILV host restriction provides a potentially unique advantage for the development of an inherently safe vaccine platform, as its inability to replicate in vertebrates eliminates the majority of the potential reactogenicity caused by live-attenuated vaccines. Therefore, EILV was investigated as an alphavirus vaccine platform.

EILV chimeras engineered to express the structural ORF of EEEV lineage I, VEEV-I AB, or CHIKV were readily rescued in *Aedes albopictus* cells, suggesting that, similar to SINV-based chimeras, EILV tolerates ORFs of other pathogenic alphaviruses (12). Previous characterization of SINV-based chimeras demonstrated that exchange of structural ORFs results in a fitness reduction both *in vitro* and *in vivo* (15–19). The reduced virus titers postelectroporation obtained for both EILV/EEEV and EILV/EEEV eRFP constructs compared to those of EILV agree with previous results and suggest a reduction in fitness *in vitro*. The reduced fitness may be due to several factors: inefficient interaction between EILV genomic RNA packaging signal(s) in the nsP ORF and the EEEV capsid, the inefficient interaction of the capsid protein and genomic RNA for assembly, reduction

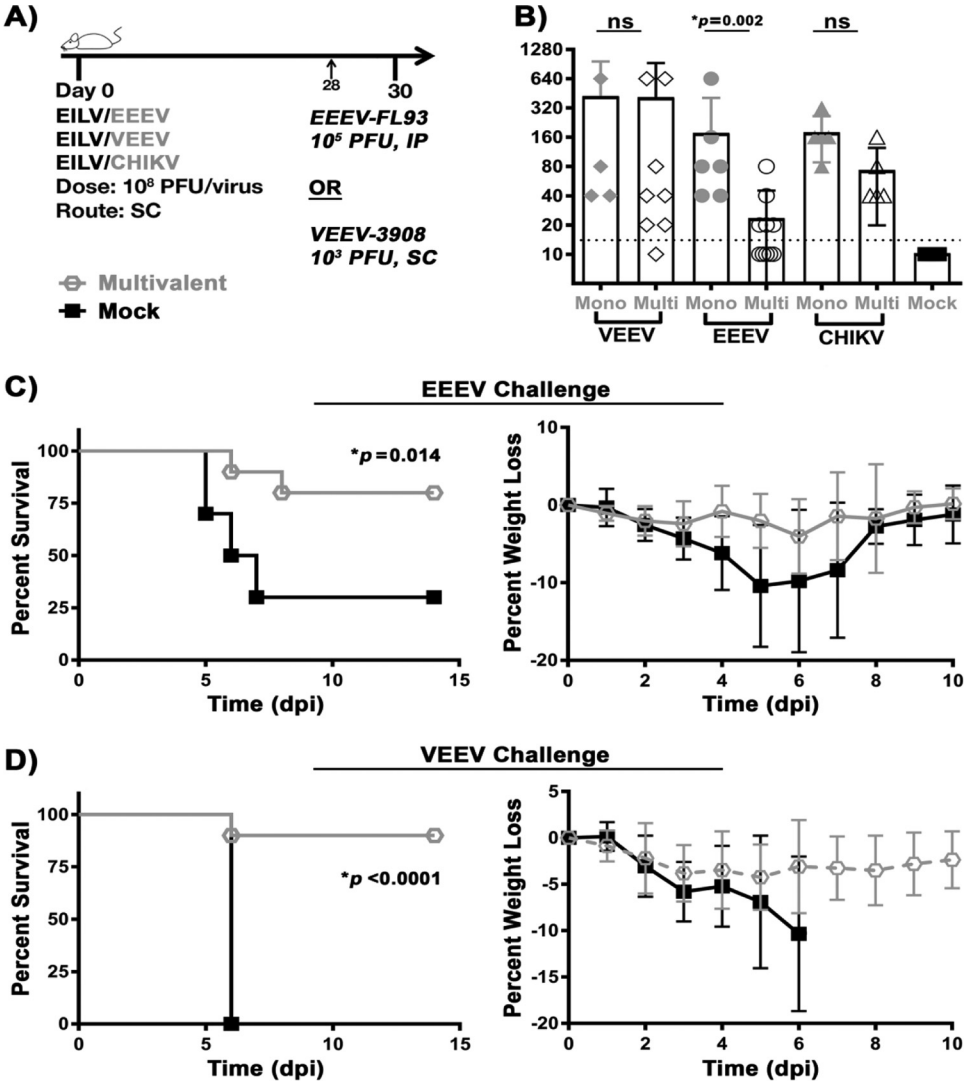

FIG 9 Immunogenicity and efficacy of blended multivalent EILV chimeras in CD-1 mice. (A) Outline of the experimental design. (B) Neutralizing antibody response measured by $PRNT_{80}$ titers following EILV/EEEV, EILV/VEEV, and EILV/CHIKV vaccination. (C and D) Percent survival and weight loss following lethal EEEV-NA (C) and VEEV-IC (D) challenge via the subcutaneous route (SC). Average $PRNT_{80}$ titers and weight losses ± SD (error bars) are shown. *, $P \leq 0.014$.

in delivery of the EILV/EEEV genome to the ribosomes, and/or reduced translation due to incompatibility with host factors (20–27). Any of these alone or in combination could delay/reduce replication and genomic RNA packaging, subsequently reducing the release of infectious virions. In contrast to EILV/EEEV chimeras, both EILV/VEEV and EILV/CHIKV yielded similar or higher virus titers than EILV, suggesting comparable or increased fitness *in vitro* in *A. albopictus* cells. One possible explanation for the increased fitness of the EILV/VEEV chimera is its ability to package VEEV subgenomic RNA, which may facilitate assembly and virion budding, thus producing higher virus titers (23). EILV/CHIKV virions do not package subgenomic RNA; however, efficient interactions outlined above may result in an increase in fitness (data not shown). Previous studies have demonstrated that EILV displays a narrow mosquito vector range due to host specialization or niche adaptation (11, 28). This narrow host range is in part due to the structural ORF. The increased fitness of EILV/VEEV and EILV/CHIKV suggests that constraints placed on EILV can be alleviated by replacing the structural ORF with that of a dual-host alphavirus. The fundamental reason(s) for reduction of/comparable/gain of fitness of EILV chimeras remains unknown and is currently being investigated.

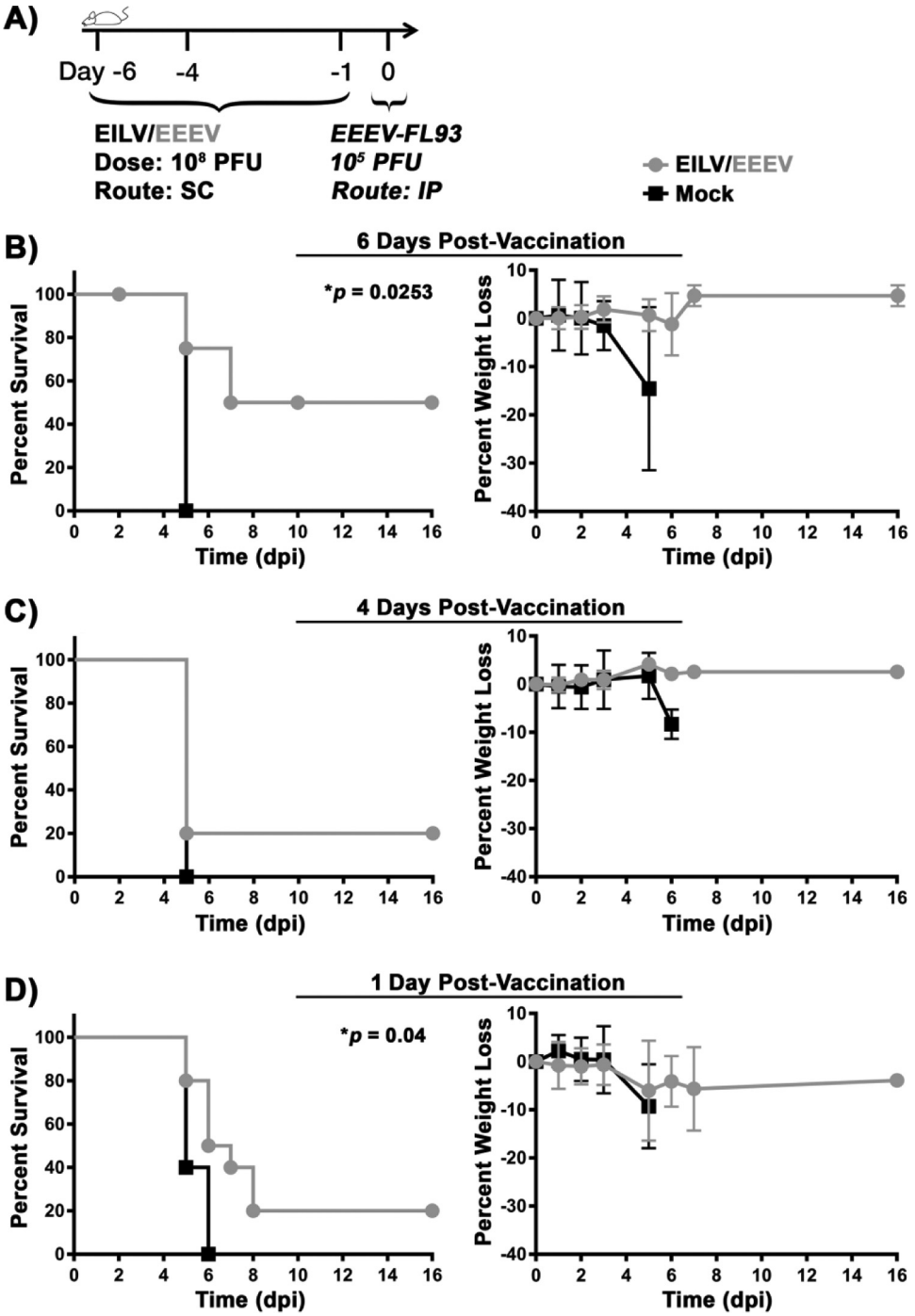

FIG 10 Kinetics of protective immunity induced by monovalent EILV/EEEV chimera in CD-1 mice. (A) Outline of the experimental design. (B to D) Percent survival and weight loss following lethal EEEV-NA challenge via the subcutaneous route (SC) at 6 (B), 4 (C), and 1 (D) day postvaccination. Average weight losses ± SD (error bars) are shown. *, $P \leq 0.04$.

The inherent safety due to the nature of the EILV host range restriction was demonstrated for the EILV/EEEV and EILV/VEEV chimeras *in vitro* and *in vivo*. These chimeras were unable to replicate in vertebrate cells or in brain tissues of infant mice. In addition, no pathological lesions were observed in the brain sections, which were indistinguishable from those of control mice injected with C7/10 cell supernatants. These data demonstrate that EILV chimeras are inherently safe, as the viruses are unable to replicate in vertebrate cells and, as a consequence, are probably unable to

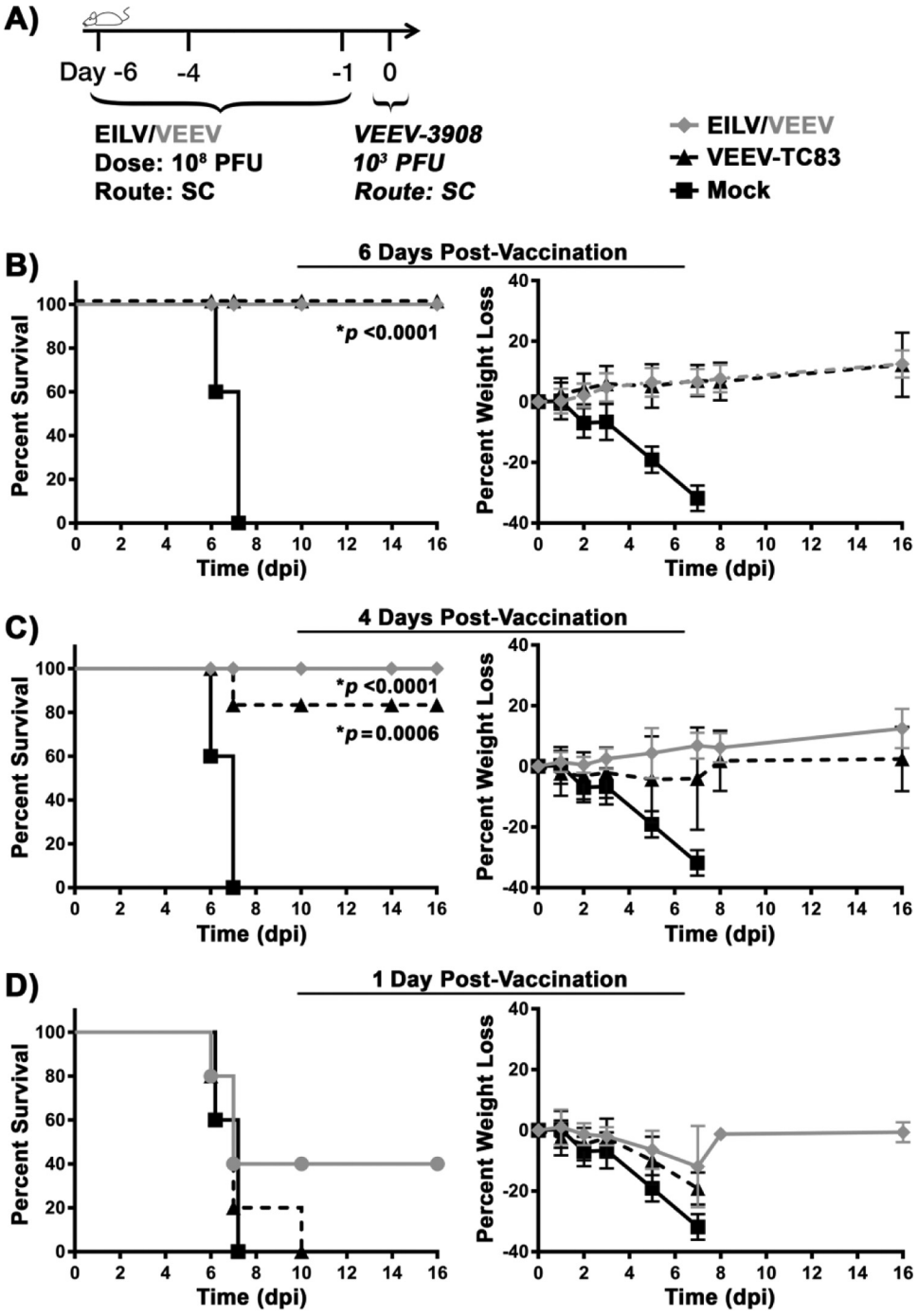

FIG 11 Kinetics of protective immunity induced by monovalent EILV/VEEV chimera in CD-1 mice. (A) Outline of the experimental design. (B to D) Percent survival and weight loss following lethal VEEV-IC challenge via the subcutaneous route (SC) at 6 (B), 4 (C), and 1 (D) day postvaccination. Average weight losses ± SD (error bars) are shown. *, $P < 0.0006$.

trigger host inflammatory processes that can proliferate quickly to damage the brain of young mice.

The EILV chimeras were immunogenic via the s.c. route, and a single dose elicited neutralizing antibodies as early as day 6 postvaccination, with maintenance of the response for at least 65 days. The kinetics and magnitude of neutralizing antibody response were faster and higher in animals vaccinated with EILV/VEEV than EILV/EEEV, which may reflect inherent differences between these chimeras, including in sub-

genomic RNA packaging, as discussed above. Surprisingly, the magnitude of the neutralizing antibody responses to the EILV-based chimeras was either comparable to or higher than that observed after either the commercial equine vaccines or the live-attenuated VEEV-TC83. More importantly, the immune responses elicited by monovalent EILV-based vaccines provided complete protection from lethal EEEV or VEEV challenge. The higher magnitude and duration of the neutralizing antibody response elicited by EILV-based vaccines may be based on the amount of antigen delivered; the amount of virus present in the inactivated commercial vaccines, which is not published by the manufacturers, could be lower. The lower immunogenicity of the commercial vaccines also could be due to poor preservation of virus epitopes following formalin treatment (29).

The mechanism of the potent immunogenicity elicited by EILV chimeras remains unknown, but several factors may contribute. (i) The EILV chimeras containing the structural ORF of dual-host alphaviruses are in essence replicons, where the attachment/entry of particles and the delivery of single-stranded genomic RNA into the cytoplasm of vertebrate cells is mediated by the heterologous structural proteins. However, once inside vertebrate cells, the translated EILV nsPs are incapable of initiating replication of the genomic RNA. Thus, this process mimics the entry of pathogenic viruses, which likely initiates recognition by multiple components of the innate immune system. The Toll-like receptors, such as TLR4, present on the cell surface can recognize envelope and/or fusion proteins of RNA viruses (30). TLR7 and TLR8 are confined to intracellular compartments, such as endosomes and the endoplasmic reticulum, and recognize single-stranded RNA (ssRNA) (31–35). In addition, retinoic acid-inducible gene I (RIG-I) and NOD-like receptor family, CARD-containing 2 (NLRC2) can also recognize ssRNA in the cytosol (36–42). The recognition of TLRs, RIG-I, and NLRC2 can signal via the MyD88, TRIF, or IPS-1 pathway to initiate the host interferon response and production of proinflammatory cytokines (26–38). (ii) The glycosylation of both the E1 and E2 glycoproteins has been shown to differ in Sindbis virus in mosquito versus vertebrate cells and could affect recognition by the TLRs and/or entry into host cells (43). (iii) The packaging of subgenomic RNA may result in increased expression of structural proteins of the pathogenic alphavirus (23). (iv) Virions are generated with surface spike structures identical to those of wild-type viruses (12). (v) The lack of virion inactivation via harsh chemical methods preserves native antigen structure and contributes to enhanced immunogenicity. (vi) The particle-to-PFU ratios of EILV chimeras may be high, resulting in vaccination with higher viral protein dose. A combination of these factors is likely to contribute to the immunogenicity of EILV-based chimeras. Additional immunogenicity studies with inactivated and native EILV chimeras may elucidate the mechanisms that underlie the nature of potent immune responses and merit further investigation.

The kinetics of immunity was also explored for both EILV-based monovalent chimeras in lethal challenge models to determine the onset of protection. EILV/EEEV provided modest protection (20 to 50%) within 6 days of a single vaccination. Surprisingly, EILV/VEEV protected 40% and 100% of the mice at 1 day and 4 or 6 days postvaccination, respectively. The protective efficacy induced by EILV/VEEV at 1 day postvaccination was greater than that of the live-attenuated VEEV-TC83, where all of the animals succumbed to disease. The mechanism of rapid EILV/VEEV protection is not known. One potential explanation is that the EILV/VEEV chimera packages subgenomic RNA, and the delivery of both genomic and subgenomic RNA induces potent innate immune responses as outlined above (23). Additionally, the VEEV structural protein ORF also can be directly translated from the delivered subgenomic RNA, resulting in endogenous antigen production and T cell presentation and subsequently faster kinetics of both humoral and cell-mediated responses. The latter is supported by the modest protection elicited by the EILV/EEEV chimera, which does not appear to package subgenomic RNA. However, this hypothesis requires further investigation.

The blended vaccine consisting of three EILV chimeras was utilized to investigate multiple alphaviruses administered in a single dose to elicit multivalent immunogenic-

ity and efficacy. A single trivalent dose of EILV/VEEV, EILV/EEEV, and EILV/CHIKV elicited neutralizing antibodies against all three viruses and provided >80% protection against VEEV and EEEV lethal challenge. The reduced efficacy against EEEV presumably was due to a lower seroconversion rate and/or lower neutralizing antibody titers. The seroconversion rates and the magnitude of $PRNT_{80}$ titers against VEEV and CHIKV in multivalent versus monovalent vaccine administrations were either comparable or slightly lower. These data suggest that there was some interference among three EILV-based chimeras. Interference has been observed with other alphavirus vaccines (44–48). However, we recently showed that this phenomenon was not observed when blended EEEV and VEEV vaccines, based on the replication-competent Isfahan virus platform, were administered to mice (49). The difference in the induction of interference may reflect the inherent biological differences in different vaccine platforms or may increase with use of three or more components in the multivalent administration.

Neutralizing antibodies are considered reliable correlates of protection against alphavirus infection, and our results are in agreement with previous reports. Several studies have suggested the potential role of nonneutralizing antibodies and/or T-cell-mediated immunity in protection; however, their role is not well studied or understood (47, 50–52). Consequently, we did not determine formal correlates of protection and used neutralizing antibody levels as a measure of immunogenicity. Both monovalent and blended EILV-based vaccine administration induced neutralizing antibody responses, produced similar $PRNT_{80}$ titers, and protected from lethal challenge. However, nonneutralizing antibodies and/or T-cell-mediated immunity likely plays a role in protection and requires further investigation.

In summary, we demonstrated that chimeras generated utilizing the mosquito-specific EILV replicated to high titers and were inherently safe, and that mono- or multivalent, single-dose administrations provided durable protection of mice from disease following lethal challenge with EEEV or VEEV. The EILV platform can be utilized to produce safe and efficacious vaccines to combat serious disease caused by the encephalitic alphaviruses.

MATERIALS AND METHODS

Cells and viruses. Vero, BHK-21, HEK-293, and C7/10 cell lines were obtained from the American Type Culture Collection (Bethesda, MD). Cell lines were propagated at 37°C or 28°C with 5% CO_2 in Dulbecco's minimal essential medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS), sodium pyruvate (1 mM), and penicillin (100 U/ml) and streptomycin (100 μg/ml). C7/10 media were additionally supplemented with 1% (vol/vol) tryptose phosphate broth (Sigma, St. Louis, MO).

VEEV-IC 3908, VEEV TC83, and EEEV-FL 93-939 were obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (UTMB) and internal collections.

Generation of cDNA clones and rescue of infectious EILV chimeras. The EILV-VEEV and -EEEV chimeras were generated by replacing the structural open reading frame (ORF) in an infectious clone of EILV with the structural protein ORFs from infectious clones of either VEEV TC-83 or EEEV-FL939 by standard cloning methods that were published previously (11, 23, 53). All EILV-EEEV or -VEEV structural chimera constructs contained enhanced green fluorescent protein (eGFP) or enhanced cherry red fluorescent protein (eRFP) under the control of an additional subgenomic promoter downstream of the nsP4 gene. Full-length cDNA clones were confirmed by ABI PRISM BigDye sequencing (Applied Biosystems).

Transcription and electroporation conditions for virus rescue. Ten micrograms of EILV chimera cDNAs was linearized using a NotI restriction site engineered immediately after the poly(A) tail. Linearized cDNAs were purified via phenol-chloroform extraction, and ~1 μg was utilized for each transcription reaction: 0.5 mM ribonucleoside triphosphate (rNTP), 0.5 mM m7G(5′)ppp(5′)G RNA cap (NEB), 0.5 μl RNase inhibitor, and 1.25 μl of SP6 polymerase (Ambion, Carlsbad, CA) in a 25-μl total volume. Transcriptions were performed for 2 h at 42°C and then placed on ice.

Cells were seeded to achieve ~70% confluence overnight. Monolayers were either trypsinized (vertebrate cells) or gently scraped (C7/10 cells) into single-cell suspensions, washed 5 times with phosphate-buffered saline (PBS), and resuspended in 450 μl or 700 μl (Vero cells only) of PBS. Cells were mixed with each transcription mixture (~10 μg of RNA), placed in 2-mm or 4-mm (Vero cells only) electroporation cuvettes, and immediately electroporated (BTX ECM-830 Square Wave electroporation system; Harvard Apparatus Inc., Holliston, MA) using the following conditions: 680 V, pulse length of 99 μs, interval between pulses of 200 ms, and number of pulses of 5. Electroporation conditions (Vero cells only) were 250 V, pulse length of 10 ms, interval between pulses of 1 s, and number of pulses of 3. Expression of eGFP or eRFP was observed with fluorescence microscopy, and supernatants containing virus were harvested at 48 hpe and stored at −80°C. All EILV chimeras expressing fluorescent reporters

were rescued in C7/10 cells, and virus supernatants were utilized in subsequent *in vitro* and *in vivo* analysis.

Virus growth, concentration, and purification. EILV chimeras were grown by infection of C7/10 cells at a multiplicity of infection (MOI) of 1 or 0.1 PFU/cell (EILV/EEEV for cryo-EM), and supernatants were harvested and clarified by centrifugation for 10 min at 1,000 × *g* at 48 h postinfection (hpi). For cryo-EM, vaccine safety, and immunogenicity studies, EILV chimeras were concentrated by polyethylene glycol (PEG) precipitation as previously described, and pellets were resuspended in TEN (0.05 M Tris-HCl [pH 7.4], 0.1 M NaCl, 0.001 M EDTA) buffer prior to purification for cryo-EM studies or in PBS for subsequent animal studies (12). To purify the PEG-precipitated virus, the resuspended virus was overlaid onto a 20 to 70% continuous sucrose gradient, and following ultracentrifugation for 1.5 h at 210,000 × *g*, the visible virus band was collected and applied to a 100-kDa Amicon filter (Millipore, Billerica, MA) and washed 5 times with TEN buffer to remove residual sucrose.

Cryo-EM and reconstruction. Cryo-EM of EILV-VEEV was performed as previously described, albeit at slightly lower magnification (12). Briefly, EILV-VEEV virions in TEN buffer were imaged on a 300-kV cryoelectron microscope with a DE-20 camera. A total of 4,760 particles imaged at 2.33 Å/pixel were damage and drift corrected by DE_process_frames and reconstructed by multipathway simulated annealing (54). The resolution was assessed to be 8.4 Å based on the criterion of an FSC of 0.143 between independently derived half-maps. Cryo-EM of EILV-EEEV was performed as previously described (55). Briefly, EILV-EEEV particles were imaged on a 200-kV cryo-electron microscope using a US4000 charge-coupled-device camera. A total of 2,145 particles with a pixel size of 2.35 Å were processed using the IMAGIC-5 software package, and the resolution was assessed at an FSC of 0.5 between the half-maps (56).

Plaque assays. Titration of EILV chimeras was performed on ~80% confluent C7/10 cell monolayers seeded overnight in six-well plates as described here, while all other viruses were titrated on Vero cells as described previously (49). Duplicate wells were infected with 0.1-ml aliquots from serial 10-fold dilutions in 1× DMEM supplemented with 2% penicillin-streptomycin, 0.4 ml of medium was added to each well to prevent cell desiccation, and virus was adsorbed for 1 h. Following incubation, the virus inoculum was removed and cell monolayers were overlaid with 3 ml of a 1:1 mixture of 2% tragacanth and 2× minimal essential medium with 5% FBS, 2% tryptose phosphate broth solution, and 2% penicillin-streptomycin. Cells were incubated at 28°C with 5% CO_2 for 3 days for plaque development, the overlay was removed, and monolayers were fixed with 3 ml of 10% formaldehyde in PBS for 30 min. Cells were stained with 2% crystal violet in 30% methanol for 5 min at room temperature; excess stain was removed under running water and plaques were counted.

$PRNT_{80}$. Serum samples were heat inactivated at 56°C for 30 min. Samples were serially diluted 2-fold in 1× DMEM, 2% FBS, gentamicin (50 μg/ml), mixed with an equal volume of 2,000 PFU/ml of VEEV TC-83 and SINV/EEEV as control viruses for VEEV and EEEV, respectively, and incubated for 1 h at 37°C. Vero cell monolayers in 6-well plates then were inoculated with 100 μl of the serum-virus mixture in triplicate. Anti-VEEV- or -EEEV-positive sera, medium only, and virus-only controls were included in the assay. Plates were incubated at 37°C and 5% CO_2 for 3 days and then fixed and stained with crystal violet as described above. $PRNT_{80}$ titers were calculated and expressed as the reciprocal of the serum dilution yielding an >80% reduction in the number of plaques.

Imaging viral infections. For infection experiments, 50% confluent monolayers were infected with EILV chimeras at an MOI of 10 PFU/cell. For electroporation experiments, ~20 to 60 μg of RNA was electroporated into ~10^7 cells via the conditions stated above. Following infection or electroporation, eGFP or eRFP expression was monitored 24 to 96 hpi. Phase-contrast and fluorescent field photographs were taken at various time points postinfection.

Mouse neurovirulence studies. All animal studies were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at UTMB (57). One- or 7-day-old newborn CD-1 outbred mice were obtained from pregnant females purchased from Charles River Laboratories (Wilmington, MA) and utilized for EILV/EEEV and EILV/VEEV studies, respectively. TC-83 virus and C7/10 cell supernatants were utilized as positive and negative controls, respectively. Animals were injected with 10 μl of EILV chimeras at 10^8 PFU/animal, C7/10 cell supernatants, or VEEV-IAB TC-83 at 10^4 PFU via the intracranial route. Animals were observed daily for signs of illness and were euthanized with the onset of neurological disease. To assess viral replication and pathology in brain tissue, cohorts of mice were euthanized at the indicated time points and brain tissues were harvested. One-half of each brain was homogenized in a 500-μl volume of PBS and titrated by plaque assay as described above, while the remaining half was fixed in 10% neutral buffered formalin (RICCA Chemical Company, Arlington, TX) and processed for histological analyses as previously described (58).

Mouse immunogenicity and efficacy studies. Adult CD-1 mice, purchased from Charles River Laboratories, were randomly distributed into their respective groups and vaccinated subcutaneously (s.c.) with EILV chimeras at 10^8 PFU/animal or with commercially available equine EEEV or VEEV vaccines. To assess immunogenicity, mice were bled at the specified time points and serum was collected by centrifugation for assay by $PRNT_{80}$ as described above. Following various incubation times, mice were challenged with either 10^5 PFU EEEV-FL93 via the intraperitoneal (i.p.) route or with 10^3 PFU VEEV-IC 3908 via the s.c. route at the specified doses and monitored daily for weight loss and clinical signs of disease. Animals were euthanized with the onset of neurological disease, consistent with prior VEEV and EEEV challenge studies.

Statistics. GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California, USA; www.graphpad.com) was utilized for statistical analysis. Survival curve analysis was determined by

performing Mantel-Cox and Gehan-Breslow-Wilcoxon test. Two-way analysis of variance (ANOVA) was performed to determine significant differences in neutralizing antibody responses and weight loss.

Accession number(s). Cryo-EM micrographs and reconstructions of EILV/EEEV and EILV/VEEV are deposited at emdatabank.org under accession numbers EMD-7004 and EMD-7003, respectively.

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F.N., J.H.E., and S.C.W. have patents on the Eilat virus technology.

REFERENCES

1. Griffin DE. 2007. Alphaviruses, p 1023–1068. *In* Fields BN, Knipe DM, Howley PM, Fields virology, 6th ed Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, PA.
2. Forrester NL, Palacios G, Tesh RB, Savji N, Guzman H, Sherman M, Weaver SC, Lipkin WI. 2012. Genome-scale phylogeny of the alphavirus genus suggests a marine origin. J Virol 86:2729–2738. https://doi.org/10.1128/JVI.05591-11.
3. Nasar F, Palacios G, Gorchakov RV, Guzman H, Da Rosa AP, Savji N, Popov VL, Sherman MB, Lipkin WI, Tesh RB, Weaver SC. 2012. Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication. Proc Natl Acad Sci U S A 109:14622–14627. https://doi.org/10.1073/pnas.1204787109.
4. Alevizatos AC, McKinney RW, Feigin RD. 1967. Live, attenuated Venezuelan equine encephalomyelitis virus vaccine. I. Clinical effects in humans. Am J Trop Med Hyg 16:762–768. https://doi.org/10.4269/ajtmh.1967.16.762.
5. Bartelloni PJ, McKinney RW, Duffy TP, Cole FE, Jr. 1970. An inactivated eastern equine encephalomyelitis vaccine propagated in chick-embryo cell culture. II. Clinical and serologic responses in man. Am J Trop Med Hyg 19:123–126. https://doi.org/10.4269/ajtmh.1970.19.123.
6. Berge TO, Banks IS, Tigertt WD. 1961. Attenuation of venezuelan equine encephalomyelitis virus by in vitro cultivation in guinea-pig heart cells. Am J Epidemiol 73:209–218. https://doi.org/10.1093/oxfordjournals.aje.a120178.
7. Cole FE, Jr, May SW, Eddy GA. 1974. Inactivated Venezuelan equine encephalomyelitis vaccine prepared from attenuated (TC-83 strain) virus. Appl Microbiol 27:150–153.
8. Maire LF, III, McKinney RW, Cole FE, Jr. 1970. An inactivated eastern equine encephalomyelitis vaccine propagated in chick-embryo cell culture. I. Production and testing. Am J Trop Med Hyg 19:119–122. https://doi.org/10.4269/ajtmh.1970.19.119.
9. Pittman PR, Makuch RS, Mangiafico JA, Cannon TL, Gibbs PH, Peters CJ. 1996. Long-term duration of detectable neutralizing antibodies after administration of live-attenuated VEE vaccine and following booster vaccination with inactivated VEE vaccine. Vaccine 14:337–343. https://doi.org/10.1016/0264-410X(95)00168-Z.
10. Sutton LS, Brooke CC. 1954. Venezuelan equine encephalomyelitis due to vaccination in man. JAMA 155:1473–1476. https://doi.org/10.1001/jama.1954.03690350015005.
11. Nasar F, Gorchakov RV, Tesh RB, Weaver SC. 2015. Eilat virus host range restriction is present at multiple levels of the virus life cycle. J Virol 89:1404–1418. https://doi.org/10.1128/JVI.01856-14.
12. Erasmus JH, Auguste AJ, Kaelber JT, Luo H, Rossi SL, Fenton K, Leal G, Kim DY, Chiu W, Wang T, Frolov I, Nasar F, Weaver SC. 2017. A chikungunya fever vaccine utilizing an insect-specific virus platform. Nat Med 23:192–199. https://doi.org/10.1038/nm.4253.
13. Voss JE, Vaney MC, Duquerroy S, Vonrhein C, Girard-Blanc C, Crublet E, Thompson A, Bricogne G, Rey FA. 2010. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. Nature 468:709–712. https://doi.org/10.1038/nature09555.
14. Zhang R, Hryc CF, Cong Y, Liu X, Jakana J, Gorchakov R, Baker ML, Weaver SC, Chiu W. 2011. 4.4 A cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. EMBO J 30:3854–3863. https://doi.org/10.1038/emboj.2011.261.
15. Atasheva S, Wang E, Adams AP, Plante KS, Ni S, Taylor K, Miller ME, Frolov I, Weaver SC. 2009. Chimeric alphavirus vaccine candidates protect mice from intranasal challenge with western equine encephalitis virus. Vaccine 27:4309–4319. https://doi.org/10.1016/j.vaccine.2009.05.011.
16. Kuhn RJ, Griffin DE, Owen KE, Niesters HG, Strauss JH. 1996. Chimeric Sindbis-Ross River viruses to study interactions between alphavirus nonstructural and structural regions. J Virol 70:7900–7909.
17. Paessler S, Fayzulin RZ, Anishchenko M, Greene IP, Weaver SC, Frolov I. 2003. Recombinant Sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic. J Virol 77:9278–9286. https://doi.org/10.1128/JVI.77.17.9278-9286.2003.
18. Wang E, Kim DY, Weaver SC, Frolov I. 2011. Chimeric Chikungunya viruses are nonpathogenic in highly sensitive mouse models but efficiently induce a protective immune response. J Virol 85:9249–9252. https://doi.org/10.1128/JVI.00844-11.
19. Wang E, Petrakova O, Adams AP, Aguilar PV, Kang W, Paessler S, Volk SM, Frolov I, Weaver SC. 2007. Chimeric Sindbis/eastern equine encephalitis vaccine candidates are highly attenuated and immunogenic in mice. Vaccine 25:7573–7581. https://doi.org/10.1016/j.vaccine.2007.07.061.
20. Frolov I, Schlesinger S. 1994. Translation of Sindbis virus mRNA: effects of sequences downstream of the initiating codon. J Virol 68:8111–8117.
21. Frolova E, Frolov I, Schlesinger S. 1997. Packaging signals in alphaviruses. J Virol 71:248–258.
22. Kim DY, Firth AE, Atasheva S, Frolova EI, Frolov I. 2011. Conservation of a packaging signal and the viral genome RNA packaging mechanism in alphavirus evolution. J Virol 85:8022–8036. https://doi.org/10.1128/JVI.00644-11.
23. Reynaud JM, Kim DY, Atasheva S, Rasalouskaya A, White JP, Diamond MS, Weaver SC, Frolova EI, Frolov I. 2015. IFIT1 differentially interferes with translation and replication of alphavirus genomes and promotes induction of type I interferon. PLoS Pathog 11:e1004863. https://doi.org/10.1371/journal.ppat.1004863.
24. Singh I, Helenius A. 1992. Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. J Virol 66:7049–7058.
25. Soderlund H, Kaariainen L. 1974. Association of capsid protein with Semliki Forest virus messenger RNAs. Acta Pathol Microbiol Scand B Microbiol Immunol 82:33–40.
26. Ulmanen I, Soderlund H, Kaariainen L. 1976. Semliki Forest virus capsid protein associates with the 60S ribosomal subunit in infected cells. J Virol 20:203–210.
27. Ventoso I. 2012. Adaptive changes in alphavirus mRNA translation allowed colonization of vertebrate hosts. J Virol 86:9484–9494. https://doi.org/10.1128/JVI.01114-12.
28. Nasar F, Haddow AD, Tesh RB, Weaver SC. 2014. Eilat virus displays a narrow mosquito vector range. Parasit Vectors 7:595. https://doi.org/10.1186/s13071-014-0595-2.
29. Fan YC, Chiu HC, Chen LK, Chang GJ, Chiou SS. 2015. Formalin inactivation of Japanese encephalitis virus vaccine alters the antigenicity and immunogenicity of a neutralization epitope in envelope protein domain III. PLoS Negl Trop Dis 9:e0004167. https://doi.org/10.1371/journal.pntd.0004167.

30. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, Finberg RW. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 1:398–401. https://doi.org/10.1038/80833.
31. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303:1529–1531. https://doi.org/10.1126/science.1093616.
32. Hardy AW, Graham DR, Shearer GM, Herbeuval JP. 2007. HIV turns plasmacytoid dendritic cells (pDC) into TRAIL-expressing killer pDC and down-regulates HIV coreceptors by Toll-like receptor 7-induced IFN-alpha. Proc Natl Acad Sci U S A 104:17453–17458. https://doi.org/10.1073/pnas.0707244104.
33. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 3:499. https://doi.org/10.1038/ni0602-499.
34. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 101:5598–5603. https://doi.org/10.1073/pnas.0400937101.
35. Wang JP, Liu P, Latz E, Golenbock DT, Finberg RW, Libraty DH. 2006. Flavivirus activation of plasmacytoid dendritic cells delineates key elements of TLR7 signaling beyond endosomal recognition. J Immunol 177:7114–7121. https://doi.org/10.4049/jimmunol.177.10.7114.
36. Cardenas WB, Loo YM, Gale M, Jr, Hartman AL, Kimberlin CR, Martinez-Sobrido L, Saphire EO, Basler CF. 2006. Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. J Virol 80:5168–5178. https://doi.org/10.1128/JVI.02199-05.
37. Faul EJ, Wanjalla CN, Suthar MS, Gale M, Wirblich C, Schnell MJ. 2010. Rabies virus infection induces type I interferon production in an IPS-1 dependent manner while dendritic cell activation relies on IFNAR signaling. PLoS Pathog 6:e1001016. https://doi.org/10.1371/journal.ppat.1001016.
38. Fredericksen BL, Keller BC, Fornek J, Katze MG, Gale M, Jr. 2008. Establishment and maintenance of the innate antiviral response to West Nile virus involves both RIG-I and MDA5 signaling through IPS-1. J Virol 82:609–616. https://doi.org/10.1128/JVI.01305-07.
39. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S. 2005. Cell type-specific involvement of RIG-I in antiviral response. Immunity 23:19–28. https://doi.org/10.1016/j.immuni.2005.04.010.
40. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441:101–105. https://doi.org/10.1038/nature04734.
41. Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M, Jr. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J Virol 82:335–345. https://doi.org/10.1128/JVI.01080-07.
42. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH, Xiang Y, Bose S. 2009. Activation of innate immune antiviral responses by Nod2. Nat Immunol 10:1073–1080. https://doi.org/10.1038/ni.1782.
43. Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD. 2003. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J Virol 77:12022–12032. https://doi.org/10.1128/JVI.77.22.12022-12032.2003.
44. Calisher CH, Sasso DR, Sather GE. 1973. Possible evidence for interference with Venezuelan equine encephalitis virus vaccination of equines by pre-existing antibody to Eastern or Western Equine encephalitis virus, or both. Appl Microbiol 26:485–488.
45. Cole FE, Jr, McKinney RW. 1971. Cross-protection in hamsters immunized with group A arbovirus vaccines. Infect Immun 4:37–43.
46. Pittman PR, Liu CT, Cannon TL, Mangiafico JA, Gibbs PH. 2009. Immune interference after sequential alphavirus vaccine vaccinations. Vaccine 27:4879–4882. https://doi.org/10.1016/j.vaccine.2009.02.090.
47. Reed DS, Glass PJ, Bakken RR, Barth JF, Lind CM, da Silva L, Hart MK, Rayner J, Alterson K, Custer M, Dudek J, Owens G, Kamrud KI, Parker MD, Smith J. 2014. Combined alphavirus replicon particle vaccine induces durable and cross-protective immune responses against equine encephalitis viruses. J Virol 88:12077–12086. https://doi.org/10.1128/JVI.01406-14.
48. Reisler RB, Gibbs PH, Danner DK, Boudreau EF. 2012. Immune interference in the setting of same-day administration of two similar inactivated alphavirus vaccines: eastern equine and western equine encephalitis. Vaccine 30:7271–7277. https://doi.org/10.1016/j.vaccine.2012.09.049.
49. Nasar F, Matassov D, Seymour RL, Latham T, Gorchakov RV, Nowak RM, Leal G, Hamm S, Eldridge JH, Tesh RB, Clarke DK, Weaver SC. 2017. Recombinant Isfahan virus and vesicular stomatitis virus vaccine vectors provide durable, multivalent, single-dose protection against lethal alphavirus challenge. J Virol 91:e01729-16. https://doi.org/10.1128/JVI.01729-16.
50. Reed DS, Lind CM, Lackemeyer MG, Sullivan LJ, Pratt WD, Parker MD. 2005. Genetically engineered, live, attenuated vaccines protect nonhuman primates against aerosol challenge with a virulent IE strain of Venezuelan equine encephalitis virus. Vaccine 23:3139–3147. https://doi.org/10.1016/j.vaccine.2004.12.023.
51. Roy CJ, Adams AP, Wang E, Leal G, Seymour RL, Sivasubramani SK, Mega W, Frolov I, Didier PJ, Weaver SC. 2013. A chimeric Sindbis-based vaccine protects cynomolgus macaques against a lethal aerosol challenge of eastern equine encephalitis virus. Vaccine 31:1464–1470. https://doi.org/10.1016/j.vaccine.2013.01.014.
52. Schmaljohn AL, Johnson ED, Dalrymple JM, Cole GA. 1982. Non-neutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis. Nature 297:70–72. https://doi.org/10.1038/297070a0.
53. Rice CM, Levis R, Strauss JH, Huang HV. 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J Virol 61:3809–3819.
54. Liu X, Jiang W, Jakana J, Chiu W. 2007. Averaging tens to hundreds of icosahedral particle images to resolve protein secondary structure elements using a Multi-Path Simulated Annealing optimization algorithm. J Struct Biol 160:11–27. https://doi.org/10.1016/j.jsb.2007.06.009.
55. Sherman MB, Weaver SC. 2010. Structure of the recombinant alphavirus Western equine encephalitis virus revealed by cryoelectron microscopy. J Virol 84:9775–9782. https://doi.org/10.1128/JVI.00876-10.
56. van Heel M, Harauz G, Orlova EV, Schmidt R, Schatz M. 1996. A new generation of the IMAGIC image processing system. J Struct Biol 116:17–24. https://doi.org/10.1006/jsbi.1996.0004.
57. National Research Council. 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, DC.
58. Plante K, Wang E, Partidos CD, Weger J, Gorchakov R, Tsetsarkin K, Borland EM, Powers AM, Seymour R, Stinchcomb DT, Osorio JE, Frolov I, Weaver SC. 2011. Novel chikungunya vaccine candidate with an IRES-based attenuation and host range alteration mechanism. PLoS Pathog 7:e1002142. https://doi.org/10.1371/journal.ppat.1002142.